Int J Clin Exp Pathol 2017;10(5):5831-5840
www.ijcep.com /ISSN:1936-2625/IJCEP0050235

Original Article

Ratio of tumor to peritumoral mitochondrial DNA content predicts prognosis of hepatitis B virus-related hepatocellular carcinoma

Shuai Wang[1*], Peng-Wen Wu[2*], Xun Wang[2], Yuan Zhou[2], Bo Dai[1], Jing-Lin Wang[2], Halmurat Obulkasim[2], Shuo Zhu[1], Xiao-Lei Shi[1,2], Yi-Tao Ding[1,2]

[1]Department of Hepatobiliary Surgery, Drum Tower Clinical Medical College of Nanjing Medical University, Nanjing, China; [2]Department of Hepatobiliary Surgery, The Affiliated Drum Tower Hospital of Nanjing University Medical School, Nanjing, China. [*]Equal contributors.

Received February 6, 2017; Accepted March 22, 2017; Epub May 1, 2017; Published May 15, 2017

Abstract: Mitochondrial DNA (mtDNA) content alteration is reported to play important roles in tumorigenesis and tumor progression. However, few studies have focused on the peritumoral tissues mtDNA copy number. In this study, we evaluated the relationship between tumor/peritumoral tissues mtDNA content and the 5-year recurrence risk of hepatocellular carcinoma (HCC). A total of 124 HBV-infected patients who had undergone curative resection for HCC were retrospectively analyzed. The mtDNA content in tumor and peritumoral tissues were determined by quantitative realtime PCR. Intratumoral mtDNA content was significantly lower than that in peritumoral tissues (P = 0.027). Compared to individuals with high mtDNA content in peritumor, those with lower mtDNA content suffered from a 2.48-fold increased risk of recurrence [95% confidence interval (CI) 1.082-5.694, P = 0.032]. The quartile analyses exhibited a significant dose relationship for this association ($P_{for\ trend}$ = 0.042). Nevertheless, we failed to draw similar significant conclusions from mtDNA content in tumor but only got a trend. Additionally, receiver operating characteristic (ROC) curve analysis presented a valuable diagnostic potential for the ratio of the mtDNA content in tumor to that in corresponding peritumoral tissues (T/P ratio) to predict HCC recurrence. This model achieved a sensitivity of 0.44 and a specificity of 0.97 with the area under ROC curve (AUC) of 0.754. HCC patients with higher T/P ratio exhibited a significantly worse 5-year recurrence-free survival. In summary, peritumoral tissues mtDNA is a potential biomarker for predicting HCC recurrence risk in HBV patients. T/P ratio could serve as a novel feasible predictor for recurrence and an independent prognosis marker in HBV-infected HCC patients.

Keywords: Mitochondrial DNA, hepatocellular carcinoma, hepatitis B, recurrence

Introduction

Primary liver cancer is the fifth most common cancer on a global scale of which the median survival is from 6 to 16 months [1]. Hepatocellular carcinoma (HCC) is the most common form which accounts for up to 90% of all malignant primary hepatoma [2]. In China, infection with hepatitis B is a major cause of chronic hepatitis, which could finally progress to HCC with a lifetime risk of 25-40% [3, 4]. There are about 55% liver cancer deaths worldwide occurring in China due to chronic hepatitis B [5]. There is an urgent need to develop reliable biomarkers that could predict clinical outcomes and guide new therapeutic strategies for HCC.

Mitochondria DNA (mtDNA) plays a crucial role in oxidative phosphorylation. The mtDNA number in a single cell varies which depends on cellular function [6]. Pathologic stimulation could alter the relative stable mtDNA content and extracellular space mtDNA released from dying cells or injured tissues can trigger host immune defenses as well [7]. It is reported that mtDNA could be transfered from non-tumor cells to tumor cells to repair the impaired mitochondrial function and simultaneously initiate tumorigenesis [8]. These high consumption of tumor cells are usually revealed by decreased level of mtDNA and could probably reflect the malignancy potential.

It is indicated that peritumoral tissues may be a favorable soil for the spreading hepatoma cells. Recurrence of HCC was associated with a unique immune/inflammation response-relat-

Table 1. Distribution of mtDNA content by patients' characteristics in tumor and peritumoral tissues

Variables	mtDNA: Median (Quartile range)		$P^{\dagger}$ value
	Peritumoral	Intratumoral	
Overall	1.90 (0.89-3.73)	0.87 (0.46-1.70)	0.027
Gender			
Male (n = 99)	1.89 (0.91-3.76)	0.93 (0.46-1.70)	0.000
Female (n = 25)	1.98 (0.57-3.65)	0.81 (0.40-1.76)	0.082
P‡	0.565	0.720	
Age (yr)			
≥ 53.4 yr (n = 62)	1.82 (0.91-3.65)	0.80 (0.46-1.70)	0.000
< 53.4 yr (n = 62)	1.98 (0.70-3.80)	0.93 (0.46-1.85)	0.000
P‡	0.486	0.459	
Smoking status			
Present (n = 44)	2.18 (0.34-5.70)	1.10 (0.26-2.76)	0.068
Absent (n = 80)	1.82 (1.00-2.85)	0.82 (0.48-1.38)	0.000
P‡	0.680	0.522	
Alcohol consumption			
Present (n = 19)	1.52 (0.63-3.75)	0.83 (0.31-1.69)	0.042
Absent (n = 105)	1.98 (0.90-3.71)	0.92 (0.46-1.75)	0.000
P‡	0.544	0.596	
Cirrhosis			
Present (n = 110)	1.94 (0.91-3.68)	0.87 (0.46-1.70)	0.000
Absent (n = 14)	1.82 (0.61-3.80)	0.93 (0.23-1.95)	0.154
P‡	0.770	0.859	
Portal hypertension			
Present (n = 19)	1.82 (0.68-3.75)	0.81 (0.31-1.69)	0.056
Absent (n = 105)	1.98 (0.91-3.71)	0.92 (0.46-1.75)	0.000
P‡	0.523	0.553	
Ascites			
Present (n = 32)	1.59 (0.68-3.49)	0.80 (0.37-1.80)	0.012
Absent (n = 92)	1.94 (0.91-3.76)	0.94 (0.46-1.70)	0.000
P‡	0.513	0.582	
Child-pugh classification			
A (n = 118)	1.90 (0.91-3.75)	0.93 (0.46-1.81)	0.000
B (n = 6)	1.31 (0.24-3.24)	0.54 (0.17-0.91)	0.262
P‡	0.328	0.091	
Number of Tumor			
Multiple (n = 29)	1.35 (0.68-2.78)	0.66 (0.31-0.99)	0.013
Solitary (n = 95)	2.00 (0.91-3.94)	0.96 (0.46-1.85)	0.000
P‡	0.052	0.065	
Tumor size			
≥ 3 cm (n = 103)	1.81 (0.88-2.98)	0.81 (0.46-1.61)	0.000
< 3 cm (n = 21)	3.75 (1.39-6.71)	1.69 (0.53-3.34)	0.048
P‡	0.023	0.019	
Microvascular_invasion			
Present (n = 46)	1.53 (0.69-2.98)	0.79 (0.46-1.36)	0.001
Absent (n = 78)	1.99 (1.06-3.92)	0.94 (0.46-1.87)	0.000
P‡	0.238	0.202	
Macrovascular_invasion			
Present (n = 20)	1.19 (0.30-2.35)	0.52 (0.28-1.53)	0.224
Absent (n = 104)	1.98 (0.96-3.76)	0.94 (0.46-1.83)	0.000
P‡	0.041	0.066	
TMN stage			

ed signature in the peritumoral noncancerous hepatic tissues [9]. For example, Zhu et al found that peritumoral macrophage colony-stimulating factors and density of macrophages had a significant promoting effect on HCC progression and recurrence [10]. However, there are few reports about the expression of mtDNA in the peritumoral hepatic tissues. In this study, we intended to investigate the putative role of mtDNA expression in the tumor and peritumoral hepatic tissues to evaluate their roles as 5-year recurrence risk factors for clinicopathologic characters of HCC patients.

Materials and methods

Patients and samples

The patients with HCC who had undergone curative resection at the Department of Hepatobiliary Surgery, the Affiliated Drum Tower Hospital of Nanjing University Medical School between May 2009 and May 2011 were analyzed retrospectively. The inclusion criteria were HBV-related HCC, TMN stage between I to III, complete follow-up data combined with required clinical information, and no preoperative anticancer treatment. According to these criteria, 124 patients were enrolled in this study. The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki. With the approval of the Institutional Ethics Committee of the Affiliated Drum Tower Hospital of Nanjing University Medical School, 124 pairs of HCC tumors and matched peritumoral samples preserved in liquid nitrogen were obtained. Peritumoral regions were defined as the periphery

I-II (n = 89)	1.99 (1.18-3.92)	0.96 (0.52-1.82)	0.000
III (n = 35)	1.16 (0.57-2.98)	0.49 (0.31-1.36)	0.058
P‡	0.014	0.028	
Recurrence			
Present (n = 66)	1.44 (0.63-3.68)	0.72 (0.33-1.67)	0.006
Absent (n = 58)	2.12 (1.36-3.79)	0.98 (0.54-1.86)	0.000
P‡	0.024	0.047	

†P, comparisons between tumor and peritumoral tissues. ‡P, comparisons between the two strata of each individual variable.

Table 2. Summary of the study population divided by recurrence situation

Variables	Recurrence (n = 66, %)	Non-Recurrence (n = 58, %)	*P* value
Gender			
Male	49 (74.2)	50 (86.2)	
Female	17 (25.8)	8 (13.8)	0.098
Age (yr, Mean±SD)	54.5±13.1	52.8±12.4	0.214
Smoking status			
Present	26 (39.4)	18 (31.0)	
Absent	40 (60.6)	40 (69.0)	0.332
Alcohol consumption			
Present	8 (12.1)	11 (19.0)	
Absent	58 (87.9)	47 (81.0)	0.291
Cirrhosis			
Present	62 (93.9)	48 (82.8)	
Absent	4 (6.1)	10 (17.2)	0.051
Portal hypertension			
Present	7 (10.6)	12 (20.7)	
Absent	59 (89.4)	46 (79.3)	0.120
Ascites			
Present	18 (27.3)	14 (24.1)	
Absent	48 (72.7)	44 (75.9)	0.691
Child-pugh classification			
A	4 (6.1)	2 (3.4)	
B	62 (93.6)	56 (96.6)	0.499
Number of Tumor			
Multiple	47 (71.2)	48 (82.8)	
Solitary	19 (28.8)	10 (17.2)	0.130
Tumor size			
≥ 3 cm	59 (89.4)	44 (75.9)	
< 3 cm	7 (10.6)	14 (24.1)	0.045
Microvascular_invasion			
Present	32 (48.5)	19 (32.8)	
Absent	34 (51.5)	39 (67.2)	0.076
Macrovascular_invasion			
Present	14 (21.2)	6 (10.3)	
Absent	52 (78.8)	52 (89.7)	0.101
TMN stage			
I-II	41 (62.1)	48 (82.9)	
III	25 (37.9)	10 (17.2)	0.011

within 1 cm of tumors adjacent to the invasion front [11].

Follow-up and clinical parameters collection

Patients were followed up in our outpatient clinic every 3 months during the first two year and at least every 6 months thereafter. If recurrence was suspected, CT scan or MRI was performed immediately. Smokers were defined for the individuals who had smoked more than 100 cigarettes in his/her lifetime. Alcohol consumption was defined as drinking more than one drink per month [12]. Portal hypertension was diagnosed according to the criteria of Barcelona Clinic Liver Cancer group defined as the presence of either esophageal varices detected by endoscopy or splenomegaly (major diameter > 12 cm) with platelet count less than 100,000/mm^3 [13]. Follow-up time is 5 years for all cases.

Measurement of mtDNA content

Total DNA was isolated from tumor and peritumoral tissue samples using the TRIzol Reagent (Life Technologies) according to the manufacturer's protocol. The relative mtDNA content was determined by quantitative real-time polymerase chain reaction (qRT-PCR) using a modified protocol as previous studies described [14]. For standardization, expression of β-actin in each sample was quantified as well, which meant the ratio of the copy number for mitochondrial ND1 gene to that of β-actin was used to determine the relative mtDNA content in this study. The sequences of primers were as follows: Forward ND1 primer: 5'-CCCT-AAAACCCGCCACATCT-3'; Rever-

Table 3. Distribution of intratumoral mtDNA content by patients' characteristics in Recurrence and Non-Recurrence group

Variables	Intratumoral mtDNA: Median (Quartile range)		*P*† value
	Recurrence	Non-Recurrence	
Overall	0.72 (0.33-1.67)	0.98 (0.54-1.86)	0.047
Gender			
Male	0.79 (0.32-1.52)	1.06 (0.52-1.87)	0.082
Female	0.58 (0.32-1.77)	0.89 (0.64-1.68)	0.322
P‡	0.859	0.982	
Age (yr)			
≥ 53.4 yr	0.58 (0.30-1.28)	0.96 (0.50-1.86)	0.079
< 53.4 yr	0.87 (0.34-1.77)	1.00 (0.61-2.04)	0.231
P‡	0.291	0.765	
Smoking status			
Present	0.39 (0.18-1.82)	2.50 (0.81-3.98)	0.008
Absent	0.80 (0.46-1.53)	0.82 (0.52-1.38)	0.736
P‡	0.136	0.004	
Alcohol consumption			
Present	0.56 (0.21-0.85)	1.12 (0.48-2.50)	0.160
Absent	0.80 (0.34-1.68)	0.96 (0.55-1.85)	0.110
P‡	0.326	0.913	
Cirrhosis			
Present	0.72 (0.31-1.67)	0.98 (0.60-1.86)	0.019
Absent	0.89 (0.49-3.23)	1.01 (0.17-1.95)	0.777
P‡	0.452	0.410	
Portal hypertension			
Present	0.34 (0.24-0.98)	0.91 (0.47-1.82)	0.272
Absent	0.79 (0.34-1.67)	1.04 (0.64-1.86)	0.061
P‡	0.441	0.558	
Ascites			
Present	0.47 (0.30-1.44)	1.12 (0.75-2.50)	0.012
Absent	0.92 (0.37-1.68)	0.98 (0.49-1.81)	0.352
P‡	0.183	0.403	
Child-pugh classification			
A	0.79 (0.34-1.68)	1.06 (0.53-1.87)	0.085
B	0.23 (0.17-0.81)	0.82 (0.82-0.82)	0.355
P‡	0.068	0.670	
Number of Tumor			
Multiple	0.52 (0.31-0.81)	0.91 (0.62-2.40)	0.023
Solitary	0.94 (0.46-1.86)	1.04 (0.53-1.85)	0.448
P‡	0.038	0.837	
Tumor size			
≥ 3 cm	0.66 (0.34-1.61)	0.82 (0.50-1.56)	0.274
< 3 cm	0.97 (0.15-2.49)	1.79 (1.15-3.98)	0.101
P‡	0.967	0.048	
Microvascular invasion			
Present	0.56 (0.34-1.28)	1.29 (0.66-1.84)	0.037
Absent	0.93 (0.31-1.97)	0.96 (0.52-1.87)	0.469
P‡	0.262	0.804	

se ND1 primer: 5'-GAGCGATGGTGAGAGCTAAGGT-3'; Forward β-actin primer: 5'-AGCGAGCATCCCCCAAAGTT-3'; Reverse β-actin primer: 5'-GGGCACGAAGGCTCATCATT-3'.

Statistical analysis

The differences in the distribution of patients' characteristics between two groups were compared using the Chi-square test or Fisher's exact test for categorical variables and the Student's t test for continuous variables. The differences of sample mtDNA content between tumor and peritumoral tissues or between recurrence group and non-recurrence group or between dichotomized patients' characteristics were compared by Wilcoxon rank-sum test. Tumor or peritumor mtDNA was analyzed as categorical variable by cutoffs at median or quartile values in the non-recurrence group. The association between tissues mtDNA content and HCC recurrence risk was evaluated by unconditional logistic regression model using univariate and multivariate analysis after adjustment for appropriate covariates in order to estimate the strength of association by odds ratio (OR) with 95% confidence interval (CI). The test for interaction between mtDNA content and patients' demographic/clinical variables on HCC recurrence risk was evaluated by including a cross-product term into the logistic regression model. Receiver operating characteristic (ROC) curve analysis was applied in predicting HCC recurrence. The discrimination accuracy of each variable was determined by the area under ROC curve (AUC) and sensitivities and specificities were calculated with the MedCalc version 14.8.1. Kaplan-

Macrovascular invasion			
Present	0.39 (0.17-1.42)	0.80 (0.43-2.78)	0.187
Absent	0.86 (0.46-1.68)	1.06 (0.56-1.87)	0.166
P‡	0.084	0.664	
TMN stage			
I-II	0.96 (0.50-1.74)	1.04 (0.52-1.86)	0.660
III	0.46 (0.29-1.08)	0.97 (0.75-2.01)	0.010
P‡	0.011	0.658	

P†, comparisons between Recurrence and Non-Recurrence. P‡, comparisons between the two strata of each individual variable.

Meier survival curve was plotted and compared with a log-rank test using the Graphpad prism version 5.01. The statistical analyses were performed using the SPSS version 19.0. All the *P* values were two sided, and P < 0.05 was considered as statistical significance.

Results

Differences of mtDNA content between HCC and peritumoral tissues

The relative content of HCC tumor and the corresponding peritomoral tissues of 124 patients were shown in **Table 1**. Overall, the mean mtDNA content in HCCs was significantly lower than that in peritumoral tissues [0.87 (0.46-1.70) vs. 1.90 (0.89-3.73) (Median (Quartile range) throughout), P = 0.027]. The mtDNA content of tumor and peritumoral tissues with clinicopathological parameters were also analyzed statistically. Both tumor and peritumoral tissues correlated with tumor size, TNM stage, and recurrence.

Characteristics of study population between recurrence group and non-recurrence group

The detailed distribution of patients' demographic characteristics was summarized in **Table 2**. No significant difference was observed between recurrence group and non-recurrence group on gender, age, smoking status, alcohol consumption, cirrhosis, portal hypertension, ascites, child-pugh classification, number of tumors, microvascular invasion, and macrovascular invasion (P = 0.098, 0.214, 0.332, 0.291, 0.051, 0.120, 0.691, 0.499, 0.130, 0.076, and 0.101, respectively), demonstrating that all these major variables were adequately matched. As expected, subjects in recurrence group had significantly poorer TNM stage and larger tumor size than cases in non-recurrence group (P = 0.011, and 0.045, respectively).

Distributions of mtDNA content in tumor and peritumor in recurrence group and non-recurrence group by patients' characteristics

The overall tumor (**Table 3**) and peritumoral tissues (**Table 4**) mtDNA content was significantly lower in recurrence group (0.72 (0.33-1.67), and 1.44 (0.63-3.68), respectively) than that in non-recurrence group (0.98 (0.54-1.86), and 2.12 (1.36-3.79), respectively). On the one hand for tumor tissues shown in **Table 3**, this differences was distinct in smokers (P = 0.008), patients with cirrhosis (P = 0.019), patients with ascites (P = 0.012), patients with multiple tumors (P = 0.023), patients with microvascular invasion (P = 0.037), and advanced HCC (TNM stage III) patients (P = 0.010). On the other hand, the significantly lower mtDNA content of peritumoral tissues in recurrence group was evident in strata such as older patients (P = 0.049), smokers (P = 0.005), cirrhotic patients (P = 0.014), patients without portal hypertension (P = 0.043), patients with ascites (P = 0.011), patients with Child-Pugh A (P = 0.046), patients with multiple tumors (P = 0.022), patients with microvascular invasion (P = 0.025), and advanced HCC (TNM stage III) patients (P = 0.007) as shown in **Table 4**.

Association between tumor and peritumoral tissues mtDNA content and HCC recurrence risk

The cutoff of median or quartile distribution of mtDNA content in recurrence group was used as a categorical variable to estimated the association between tumor and peritumoral tissues mtDNA content and HCC risk through unconditional logistic regression model. As shown in **Table 5** for tumor tissues, patients with a lower level of tumor mtDNA content (≤ 0.98) revealed a significantly increased recurrence risk with a crude OR of 2.143 (95% CI 1.032-4.448, P = 0.041) in univariate analysis. Patients in recurrence group had low tumor mtDNA content, but not significant, when assessed in a multivariate analysis adjusting for smoking status, cirrhosis, ascites, number of tumors, microvascular inva-

Table 4. Distribution of peritumoral tissues mtDNA content by patients' characteristics in Recurrence and Non-Recurrence group

Variables	Peritumoral mtDNA: Median (Quartile range)		*P*† value
	Recurrence	Non-Recurrence	
Overall	1.44 (0.63-3.68)	2.12 (1.36-3.79)	0.024
Gender			
Male	1.53 (0.68-3.37)	2.26 (1.36-3.92)	0.054
Female	1.16 (0.30-4.22)	1.98 (1.86-3.45)	0.322
P‡	0.676	0.910	
Age (yr)			
≥ 53.4 yr	1.16 (0.51-3.08)	1.99 (1.44-3.79)	0.049
< 53.4 yr	1.68 (0.68-3.73)	2.27 (1.36-3.85)	0.194
P‡	0.312	0.832	
Smoking status			
Present	0.79 (0.24-3.76)	4.78 (1.88-9.24)	0.005
Absent	1.59 (0.69-3.49)	1.98 (1.36-2.72)	0.597
P‡	0.088	0.005	
Alcohol consumption			
Present	1.08 (0.34-1.21)	2.71 (1.52-4.77)	0.069
Absent	1.59 (0.66-3.76)	1.99 (1.36-3.65)	0.089
P‡	0.276	0.736	
Cirrhosis			
Present	1.44 (0.63-3.68)	2.13 (1.52-3.85)	0.014
Absent	1.49 (0.72-4.74)	2.27 (0.61-3.80)	1.000
P‡	0.788	0.446	
Portal hypertension			
Present	0.68 (0.24-2.85)	1.90 (1.17-3.88)	0.176
Absent	1.52 (0.68-3.76)	2.26 (1.52-3.72)	0.043
P‡	0.298	0.539	
Ascites			
Present	0.69 (0.57-2.53)	2.31 (1.70-4.77)	0.011
Absent	1.73 (0.68-3.88)	1.99 (1.36-3.73)	0.313
P‡	0.169	0.364	
Child-pugh classification			
A	1.53 (0.68-3.68)	2.13 (1.36-3.88)	0.046
B	0.44 (0.24-3.17)	2.48 (1.99-2.98)	0.355
P‡	0.179	0.701	
Number of Tumor			
Multiple	1.00 (0.46-1.65)	2.35 (1.27-4.49)	0.022
Solitary	2.00 (0.68-4.02)	2.13 (1.52-3.88)	0.356
p‡	0.059	0.934	
Tumor size			
≥ 3 cm	1.36 (0.68-2.99)	1.98 (1.36-2.82)	0.110
< 3 cm	1.82 (0.13-5.71)	3.88 (2.36-8.94)	0.179
P‡	0.975	0.068	
Microvascular_invasion			
Present	1.08 (0.78-2.73)	2.36 (1.35-4.01)	0.025
Absent	1.91 (0.54-4.21)	1.99 (1.36-3.75)	0.445

sion, and TMN stage (P = 0.079). As to peritumoral tissues (**Table 6**), patients with low mtDNA content had a 2.300-fold increased risk of recurrence (95% CI 1.103-4.798, P = 0.026) compared to those with a high level of mtDNA content. We could also observe a significant association after adjustment for age, smoking status, cirrhosis, portal hypertension, ascites, child-pugh classification, tumor numbers, microvascular invasion, and TMN stage (2.48 (1.082-5.694), P = 0.032). In quartile analysis, the results failed to confirmed that recurrence risk increased with decreased intratumoral mtDNA content. The $P_{for\ trend}$ value in univariate analyses were 0.040, but were 0.097 after adjustment for covariates. As to peritumoral tissues, the quartile analyses exhibited a significant dose-dependent effect for this association in multivariate analysis ($P_{for\ trend}$ = 0.042).

Prognostic prediction of the mtDNA content ratio of tumor to peritumoral tissues for recurrence

We further evaluated whether tumor or peritumoral tissues mtDNA content could predict HCC recurrence. As shown in **Figure 1A**, **1B**, the AUC of ROC curve for tumor, and peritumoral tissues for recurrence was 0.606 (95% CI, 0.514-0.692, P = 0.038), and 0.626 (95% CI, 0.534-0.711, P = 0.013), respectively, with cut-off points at 0.579, and 1.215, respectively. Then, we calculated the ratio of the mtDNA content in the tumor to that in the corresponding peritumoral tissues (T/P ratio). Interestingly, the AUC of T/P ratio was 0.754 (95% CI, 0.669-0.827, P < 0.001) with the cut-off point at 0.596 (**Figure 1C**).

p‡	0.287	0.673	
Macrovascular_invasion			
Present	0.57 (0.24-2.53)	1.81 (1.24-5.40)	0.099
Absent	1.59 (0.68-3.76)	2.13 (1.52-3.88)	0.120
P‡	0.066	0.507	
TMN stage			
I-II	2.00 (0.94-3.93)	1.99 (1.25-3.88)	0.730
III	0.68 (0.30-1.77)	2.31 (1.82-3.93)	0.007
p‡	0.006	0.537	

P†, comparisons between Recurrence and Non-Recurrence. P‡, comparisons between the two strata of each individual variable.

Prognostic analysis for tumor/peritumoral mtDNA content and T/P ratio in HCC patients

According to the cut-off points above, we dichotomized patients into two subgroups with high or low mtDNA content or T/P ratio. As to the taxonomy upon mtDNA content, Kaplan-Meier survival function analysis showed that patients with low mtDNA content had a shorter recurrence free survival (RFS) than did those with high mtDNA content in tumor and peritumoral tissues (log-rank P = 0.010 and 0.002, respectively, **Figure 2A**, **2B**). Next, we further used the Kaplan-Meier method to analyze patient survival according to the taxonomy upon T/P ratio. The data showed that the 5-year RFS rate of the low T/P ratio group was significantly higher than those with high T/P ratio (P < 0.001) (**Figure 2C**). Therefore, our research displayed that low tumor/peritumoral mtDNA content or high T/P ratio were correlated with a poor survival.

Discussion

In the present study, we compared the mtDNA content between HCC intratumoral and peritumoral tissues and evaluated the association between HCC recurrence risk in a clinic-based cohort of HBV-infected patients and mtDNA content in tumor and peritumor. We demonstrated that the intratumoral mtDNA level was lower than that in peritumoral tissues, and a low level of mtDNA content in peritumoral tissues was significantly associated with an increased risk of recurrence in a dose-dependent manner. Our results also indicated that T/P ratio could be a reliable biomarker for predicting HCC recurrence.

It is well-known that mitochondria take part in many vital physiological processes, such as energy production, metabolism, and so on. In recent years, mtDNA has been demonstrated alterable in tumors and plays important role in tumorigenic process [15]. Precious studies about relationships between tissues mtDNA and HCC are few and relatively simple. In the present study, we found that the mtDNA in HCC was significantly decreased compared to that of peritumoral tissues. This significant difference still exists in stratified analysis by clinicopathological parameters except for some subgroup with small sample sizes. Since we found that there was a close association between tumor or periumoral tissues mtDNA content and recurrence, it is necessary to further elucidate the deep correlations and prognostic significance.

Recurrence is a major problem for curative resection of HCC which usually herald a worse prognosis compared with late phase recurrence. In this study, the multivariate analysis failed to reveal a significant association between intratumoral mtDNA content and HCC recurrence risk through unconditional logistic regression model. Patients with lower tumor mtDNA content had a tendency to have recurrence, but not significant (P = 0.079, **Table 5** with the median as cutoff value). However, we identified peritumoral tissues mtDNA content as independent prognostic factor for HCC recurrence in multivariable analysis (P = 0.032, **Table 6** with the median as cutoff value). As far as we know, the predicting significance of peritumoral tissues mtDNA content alone have not been reported in the past. So we further evaluated discrimination accuracy through ROC curve and found that T/P ratio (AUC > 0.7) could provided diagnostic value for HCC recurrence rather than tumor or peritumoral tissues mtDNA content alone (AUC < 0.7). This result indicated that though the decreased mtDNA content in HCC which has been reported in many studies [16, 17], it could not directly applied to prognostic analysis since the baseline value of mtDNA content differs from patients by nature. T/P ratio reflected the mtDNA contents gap between tumor and tumoral tissues which eliminates the individual differences to some extent and could be considered for further researches. This could be the reason why T/P

Table 5. The association between tumor mtDNA content and HCC recurrence risk

mtDNA	Non-Recurrence	Recurrence	Univariate		Multivariate†	
			OR (95% CI)	*P* value	OR (95% CI)	*P* value
By median						
> 0.98	29	21	1.000		1.000	
≤ 0.98	29	45	2.143 (1.032-4.448)	0.041	2.032 (0.920-4.489)	0.079
By quartile						
1.86 >	14	12	1.000		1.000	
1.86-	15	9	0.700 (0.226-2.167)	0.536	0.643 (0.186-2.223)	0.485
0.98-	15	17	1.322 (0.468-3.732)	0.598	1.280 (0.405-4.049)	0.674
≤ 0.54	14	28	2.333 (0.856-6.362)	0.098	2.010 (0.670-6.034)	0.213
$P_{for\ trend}$				0.040		0.097

†Adjusted for smoking status, cirrhosis, ascites, number of tumor, microvascular invasion, and TMN stage.

Table 6. The association between peritumoral mtDNA content and HCC recurrence risk

mtDNA	Non-Recurrence	Recurrence	Univariate		Multivariate†	
			OR (95% CI)	*P* value	OR (95% CI)	*P* value
By median						
> 2.12	29	20	1.000		1.000	
≤ 2.12	29	46	2.300 (1.103-4.798)	0.026	2.48 (1.082-5.694)	0.032
By quartile						
> 3.79	14	14	1.000		1.000	
3.79-	15	6	0.400 (0.120-1.331)	0.135	0.349 (0.091-1.342)	0.126
2.12-	15	14	0.933 (0.330-2.638)	0.896	0.947 (0.293-3.053)	0.927
≤ 1.36	14	32	2.286 (0.865-6.037)	0.095	2.355 (0.741-7.488)	0.147
$P_{for\ trend}$				0.028		0.042

†Adjusted for age, smoking status, cirrhosis, portal hypertension, ascites, Child-pugh classification, number of tumor, microvascular invasion, TMN stage.

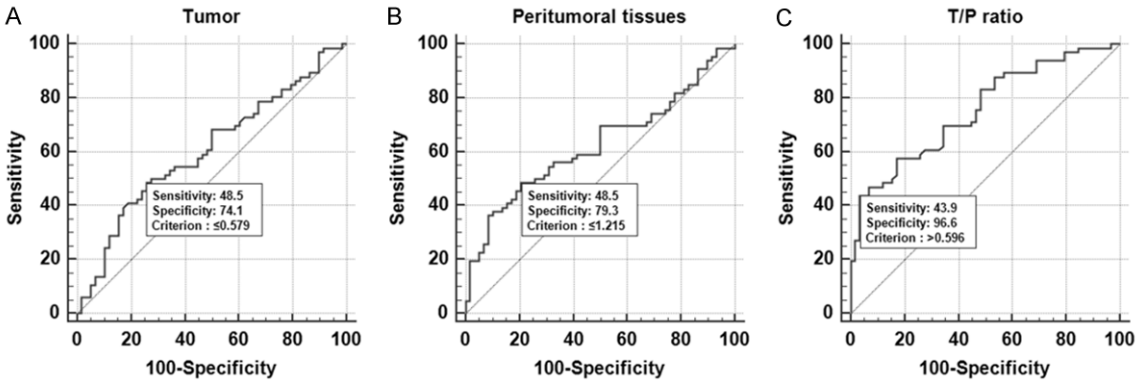

Figure 1. ROC curve of intratumoral mtDNA content, peritumorol tissues mtDNA content, and T/P ratio for HCC recurrence. A. Intratumoral mtDNA content had an AUC of 0.606 and cut-off of 0.579 for HCC recurrence. B. Peritumorol tissues mtDNA content had an AUC of 0.626 and cut-off of 1.215 for HCC recurrence. C. T/P ratio had an AUC of 0.754 and cut-off of 0.596 for HCC recurrence.

ratio acted better in predicting HCC recurrence.

There are many reasons causing the reduction in the mtDNA content. It is common recognized

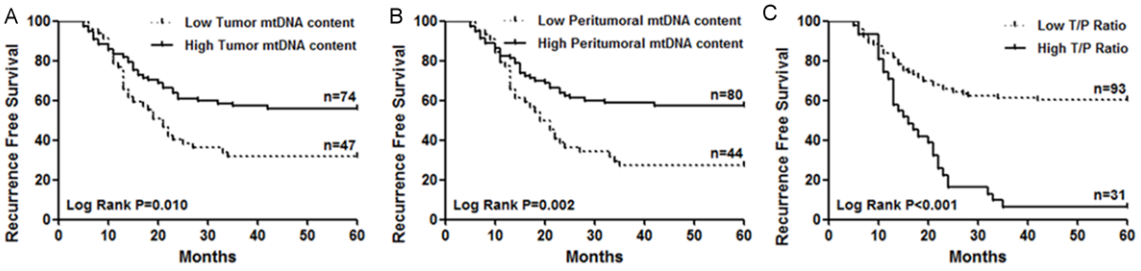

Figure 2. Kaplan-Meier estimates of RFS in HBV-related HCC patients by intratumoral, peritumoral mtDNA content and T/P ratio mtDNA content. A. RFS of patients with tumor mtDNA content > 0.579 was signifcantly longer than those with tumor mtDNA content ≤ 0.579 (P = 0.010, log-rank test). B. RFS of patients with peritumoral mtDNA content > 1.215 was markedly longer than those with peritumoral mtDNA content ≤ 1.215 (P = 0.003, log-rank test). C. RFS of patients with T/P ratio ≤ 0.596 was signifcantly longer than those with T/P ratio > 0.596 (P < 0.001, log-rank test).

that there is a unique displacement loop (D-loop) in the non-coding region which controls replication and transcription of mtDNA [18]. Most of the mtDNA mutations are found to be located in this D-loop region [19]. It was suggested that the down-regulation of mtDNA content may result from mutations in the D-loop region [16]. What's more, It was reported that the decrease in the mtDNA content HCC may result from the altered expression of genes due to impairment in mitochondrial biogenesis, such as mitochondrial single-stranded DNA binding protein [19, 20]. Whatever the cause, the decrease in mtDNA content could further promote tumorigenesis and progression. Cells depleted of mtDNA were able to enhance the anti-apoptosis [21] and invasive activities [22]. In addition, Simonnet H, et al reported that the decreased oxidative phosphorylation capacity induced by mitochondrial impairment favored faster growth or increased invasiveness of renal cell carcinomas [23]. A widely accepted view is that mitochondria, as the major source of reactive oxygen species (ROS) [24], were altered in gene expression and enhanced in production of ROS on account of deficiency in oxidative phosphorylation due to decreased mtDNA content, all of which could lead to nuclear DNA damage and malignant cellular transformation [25]. Corresponding to these studies, we found that HBV-related HCC patients with lower mtDNA content are more vulnerable to recurrence, which indicated greater malignant behavior.

In conclusion, this study revealed that the decreased mtDNA content in peritumoral tissues was significantly associated with the increased risk of recurrence in HCC patients with chronic HBV infection. Moreover, T/P ratio could serve as a novel feasible predictor for recurrence risk in HBV-infected HCC patients. Larger cohorts are still needed to confirm these results and explore the underlying molecular mechanisms in order to further explain the clinical relevance of mtDNA content determination in HCC.

Acknowledgements

This project was supported by grants from the National Natural Science Fund of China (NSFC) (81670566).

Disclosureof conflict of interest

None.

Address correspondence to: Drs. Xiao-Lei Shi and Yi-Tao Ding, Department of Hepatobiliary Surgery, The Affiliated Drum Tower Hospital of Nanjing University Medical School, No. 321 Zhongshan Road, Nanjing 210008, Jiangsu Province, China. Tel: 86-25-83304616; Fax: 86-25-83317016; E-mail: njsxl2005@126.com (XLS); njytding@163.com (YTD)

References

[1] Lin L, Amin R, Gallicano GI, Glasgow E, Jogunoori W, Jessup JM, Zasloff M, Marshall JL, Shetty K, Johnson L, Mishra L, He AR. The STAT3 inhibitor NSC 74859 is effective in hepatocellular cancers with disrupted TGF-beta signaling. Oncogene 2009; 28: 961-972.

[2] Grandhi MS, Kim AK, Ronnekleiv-Kelly SM, Kamel IR, Ghasebeh MA, Pawlik TM. Hepatocellular carcinoma: from diagnosis to treatment. Surg Oncol 2016; 25: 74-85.

[3] Zhang Y, Zhu SL, Chen J, Li LQ. Meta-analysis of associations of interleukin-28B polymorphisms rs8099917 and rs12979860 with development of hepatitis virus-related hepatocellular carcinoma. Onco Targets Ther 2016; 9: 3249-3257.
[4] Kao JH, Chen DS. Global control of hepatitis B virus infection. Lancet Infect Dis 2002; 2: 395-403.
[5] McGlynn KA, London WT. Epidemiology and natural history of hepatocellular carcinoma. Best Pract Res Clin Gastroenterol 2005; 19: 3-23.
[6] Miller FJ, Rosenfeldt FL, Zhang C, Linnane AW, Nagley P. Precise determination of mitochondrial DNA copy number in human skeletal and cardiac muscle by a PCR-based assay: lack of change of copy number with age. Nucleic Acids Res 2003; 31: e61.
[7] Chen GY, Nuñez G. Nunez, Sterile inflammation: sensing and reacting to damage. Nat Rev Immunol 2010; 10: 826-837.
[8] Rebbeck CA, Leroi AM, Burt A. Mitochondrial capture by a transmissible cancer. Science 2011; 331: 303.
[9] Budhu A, Forgues M, Ye QH, Jia HL, He P, Zanetti KA, Kammula US, Chen Y, Qin LX, Tang ZY, Wang XW. Prediction of venous metastases, recurrence, and prognosis in hepatocellular carcinoma based on a unique immune response signature of the liver microenvironment. Cancer Cell 2006; 10: 99-111.
[10] Zhu XD, Zhang JB, Zhuang PY, Zhu HG, Zhang W, Xiong YQ, Wu WZ, Wang L, Tang ZY, Sun HC. High expression of macrophage colony-stimulating factor in peritumoral liver tissue is associated with poor survival after curative resection of hepatocellular carcinoma. J Clin Oncol 2008; 26: 2707-2716.
[11] Zhuang PY, Wang JD, Tang ZH, Zhou XP, Yang Y, Quan ZW, Liu YB, Shen J. Peritumoral neuropilin-1 and VEGF receptor-2 expression increases time to recurrence in hepatocellular carcinoma patients undergoing curative hepatectomy. Oncotarget 2014; 5: 11121-11132.
[12] Li L, Hann HW, Wan S, Hann RS, Wang C, Lai Y, Ye X, Evans A, Myers RE, Ye Z, Li B, Xing J, Yang H. Cell-free circulating mitochondrial DNA content and risk of hepatocellular carcinoma in patients with chronic HBV infection. Sci Rep 2016: 6: 23992.
[13] Llovet JM, Brú C, Bruix J. Prognosis of hepatocellular carcinoma: the BCLC staging classification. Semin Liver Dis 1999; 19: 329-338.
[14] Xing J, Chen M, Wood CG, Lin J, Spitz MR, Ma J, Amos CI, Shields PG, Benowitz NL, Gu J, de Andrade M, Swan GE, Wu X. Mitochondrial DNA content: its genetic heritability and association with renal cell carcinoma. J Natl Cancer Inst 2008; 100: 1104-1112.
[15] Wallace DC. Mitochondria and cancer. Nat Rev Cancer 2012; 12: 685-698.
[16] Lee HC, Li SH, Lin JC, Wu CC, Yeh DC, Wei YH. Somatic mutations in the D-loop and decrease in the copy number of mitochondrial DNA in human hepatocellular carcinoma. Mutat Res 2004; 547: 71-78.
[17] Cuezva JM, Krajewska M, de Heredia ML, Krajewski S, Santamaría G, Kim H, Zapata JM, Marusawa H, Chamorro M, Reed JC. The bioenergetic signature of cancer: a marker of tumor progression. Cancer Res 2002; 62: 6674-6681.
[18] Attardi G, Schatz G. Biogenesis of mitochondria. Annu Rev Cell Biol 1988; 4: 289-333.
[19] Hsu CC, Lee HC, Wei YH. Mitochondrial DNA alterations and mitochondrial dysfunction in the progression of hepatocellular carcinoma. World J Gastroenterol 2013; 19: 8880-8886.
[20] Yin PH, Lee HC, Chau GY, Wu YT, Li SH, Lui WY, Wei YH, Liu TY, Chi CW. Alteration of the copy number and deletion of mitochondrial DNA in human hepatocellular carcinoma. Br J Cancer 2004; 90: 2390-2396.
[21] Chandel NS, Schumacker PT. Cells depleted of mitochondrial DNA (rho0) yield insight into physiological mechanisms. FEBS Lett 1999; 454: 173-176.
[22] Purnomosari D, Pals G, Wahyono A, Aryandono T, Manuaba TW, Haryono SJ, van Diest PJ. BRCA1 and BRCA2 germline mutation analysis in the Indonesian population. Breast Cancer Res Treat 2007; 106: 297-304.
[23] Simonnet H, Alazard N, Pfeiffer K, Gallou C, Béroud C, Demont J, Bouvier R, Schägger H, Godinot C. Low mitochondrial respiratory chain content correlates with tumor aggressiveness in renal cell carcinoma. Carcinogenesis 2002; 23: 759-768.
[24] Murphy MP. How mitochondria produce reactive oxygen species. Biochem J 2009; 417: 1-13.
[25] Lee HC, Yin PH, Lu CY, Chi CW, Wei YH. Increase of mitochondria and mitochondrial DNA in response to oxidative stress in human cells. Biochem J 2000; 348 Pt 2: 425-432.